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Abstract

Some critical experiments have been carried out on the microspectrophotometry using the lymphocytes of a mouse, stained with Feulgen reaction, revealing that most reliable value can be attained by illuminating the material with a small spot-light and integrating the area surrounded by the extinction curve drawn by tracing along the diameter of the smeared and fixed cell.

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QUANTITATIVE STUDIES OF NUCLEIC ACID IN THE CELL BY MICROSPECTROPHOTOMETRY

I. THE CRITIQUE AND IMPROVEMENT OF THE MICROSPECTROPHOTOMETRY

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One of the most important themes in cytology must be the problem of nucleus as the bearer of genetical characteristics and the organizing center of the cell. Biochemical researches of nucleic acids are increasing in their validity in the field of genetics and it is now the time when the old morphologic investigations of chromosomes are rescrutinized under the light of biochemical aspects which have developed to such a high degree in the last decade. The recent cytochemical and cytophysical researches make it possible to connect the old with new observations¹. Thus at present it has become possible to search the close relation between the chromosome number and the desoxypentose nucleic acid (DNA) content in a cell on the established background of biochemical and morphologic data^{1,7}. Especially, the quantitative estimation of nucleic acids by means of microspectrophotometer (MSP), first introduced by CASPERSSON³ and is improved by many reseachers^{8,13}, has made a great contribution to this field. However, the slight differences in the method used have led to quit different experimental results causing the conflicting opinions, i. e. some authors^{2,4,5,6,7,11,15,16,17,18} assert the DNA constancy in each cell species but others^{19,20,21} disagree to this views, though the increased DNA content in some period of cell division is now a general conception. This confusion seems to be the results of incompleteness of the method in determining the DNA content in each cell accurately. For this reason the author tried with the aid of SENO to improve the method of microspectrophotometry checking the obtained values by comparing with those obtained by the chemical analysis. Thus as reported in the previous papers²²⁻²⁵ the hemoglobin values obtained by the improved microspectrophotometry coincided well with the values calculated from the number of red cells and hemoglobin content in a given unit volume of blood.

In this paper the author presents the data on the DNA content of

lymphocytes estimated by the improved MSP comparing with those obtained by the method described by various authors.

MATERIALS AND METHODS

Lymphocytes from spleen and circulating blood of mice served as materials. These were stamped or smeared, fixed with acetic alcohol²⁶, stained by Feulgen nuclear reaction^{27,28} and the DNA content in each nucleus was estimated by microspectrophotometry. The MSP used by the author for the present study is of Olympus Co., Tokyo, attached with micro-sliding stage designed by SENO and the autorecorecording apparatus of Yokogawa Co., Tokyo. In this apparatus the illuminating area can be changed from 0.67μ to about 100μ in diameter.

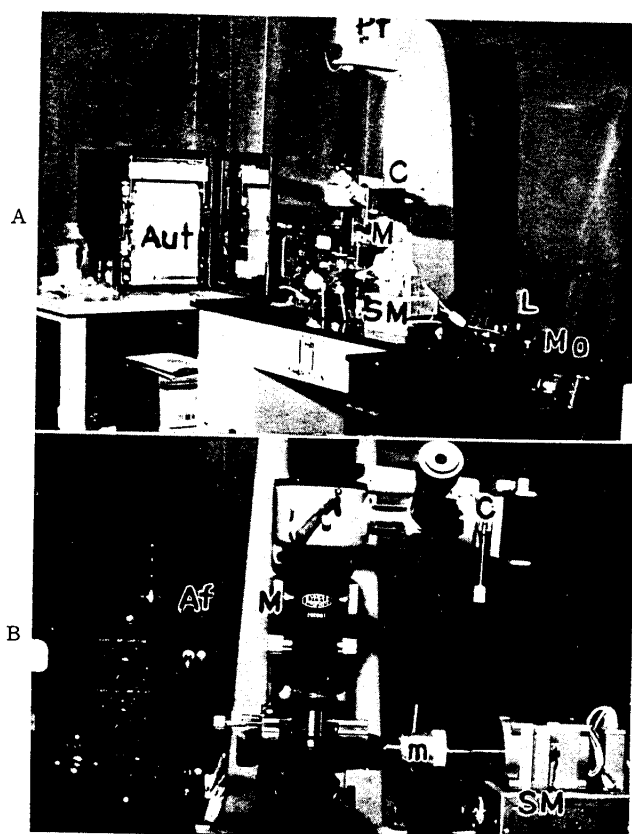


Fig. 1. The Photographs of the Microspectrophotometer.

A is whole set from forward. B is microsliding stage from forward. L: light source, Mo: monochrometer, m: micrometer, SM: synchronous motor, M: microscope, C: camera, Pt: photo-tube, Af: amplifier, Aut: autorecording apparatus.

In the first figure the photos of this apparatus, whole set from forward (A), and micro-sliding stage from forward (B) are demonstrated. The diagram of light path is seen in Figure 2.

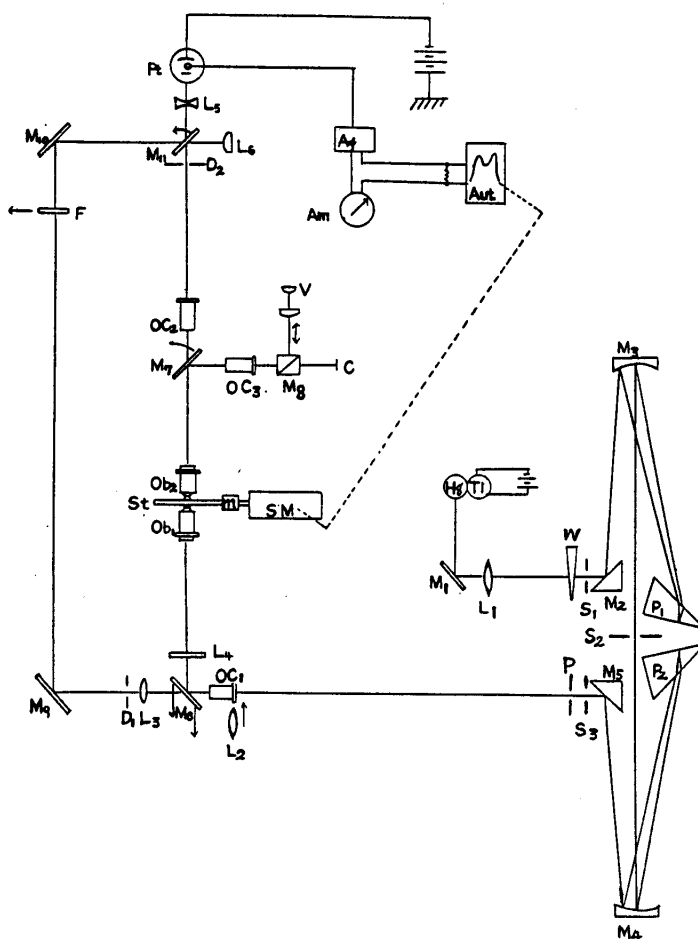


Fig. 2. The diagram of the light path of the microspectrophotometer.

Hg: high pressure-mercury lamp, Tl: tangsten lamp, W: optical wedge, M₃-M₄: monochromator, P: pin-hole, OC₁-Ob₁: condenser, Ob₂-OC₂: microscope, m: micrometer, SM: synchronous motor, C: camera, M₉-M₁₀: by-path, Pt: photo-tube, Af: amplifier, Am: ammeter, Aut: autorecording apparatus, F: filter.

As the light source, high-pressure-mercury lamp (Hg) (2400 Å to 5770 Å) or tangsten lamp (6 v, 42 w) (Tl) designed specifically for this apparatus so as the picture of filament to fit to the space of the slit which

leads the light into the first prism. A monochrom-light in the range of 1.5 Å to 18 Å can be obtained passing through two prisms (P_1 and P_2), four reflecting surfaces (M_2 , M_3 , M_4 , and M_5) and two diaphragms (S_1 and S_2). The monochromatic light leaving the last diaphragm (S_3) passes through a pin-hole (P) (1 to 2 mm. in diameter), and is introduced into the microscope through the condenser. By the condenser Ob_1 combined with L_2 this light spot is converged on to a spot-light, about $100\ \mu$ in diameter or a smaller light-spot of $1.5\ \mu$ in diameter with reflecting objective²⁹ $\times 175$ (Ob_1) only or $0.67\ \mu$ with an ocular $\times 5$ (OC_1) combined with Ob_1 . Thus the light-spot illuminating a small area of the material is led into the photo-tube (Pt) passing through the objective (Ob_2) and the diaphragm (D_2) set just in front of the photo-tube. The spaces between the condenser and objective slide and between the objective lens and the material is filled with the immersion fluid, glycerol. The picture of the cell can be seen with the viewer (V) or taken by camera (C) with the slanted mirror M_7 and the condenser Ob_1 combined with lens L_2 . By setting up the slanted mirror the light is introduced into the photo-tube. The electric current led from photo-bube is taken and amplified by the amplifier (Af) and is led into both the autorecording apparatus (Aut) and ammeter (Am). The stage (St) is made to be movable by the attached micrometer (m) and the connected synchronous motor (SM), $1\ \mu$ move for 7 seconds, whose movement is synchronized to that of autorecording apparatus.

EXPERIMENTS AND RESULTS

Using the apparatus and the cells having Feulgen reaction described above, the estimation of DNA has been carried out. As indicated in Fig. 3. the nucleus stained with Feulgen reaction has the absorption maximum at 5600 Å. Then the estimation of DNA has been carried out with this light. The calculation of the relative value of DNA contents of the nucleus has been done by multiplying the extinction E obtained at a limited area by the whole area of the nucleus. In Table 1, the values obtained by multiplying the extinction at the central area of the nucleus, 0.67 or $1.0\ \mu$ in diameter, by the whole area of the nucleus are presented. In this case Ob_2 combined with OC_2 is used as the microscope. The light passing through the central part of the nucleus, $1.0\ \mu$ in diameter, is selectively introduced into photo-tube by diaphragm D_2 . Illumination was done in three different ways: illumination on the whole area of the microscope field (Ob_1 combined with L_2), the area of $1.5\ \mu$ in diameter (Ob_1 only) or $0.67\ \mu$ in diameter (Ob_1 combined with OC_1). Thus the relative value of

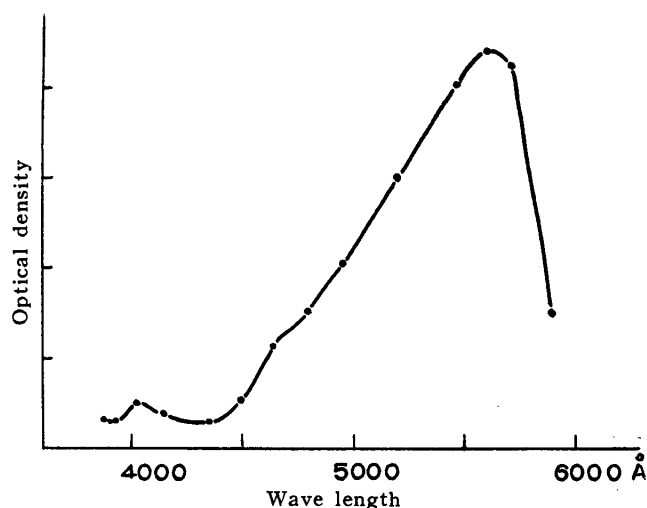


Fig. 3. Absorption spectrum of the nucleus of a lymphocyte stained by Feulgen nuclear reaction. The curve has been drawn by the MSP with a spot-light of 1.5μ in diameter, using the tungsten lamp as a light source.

DNA contents of the nucleus, C_Q can be calculated by the following formula

$$C_Q = r^2 E \quad (1)$$

where r is the radius of the round nucleus and E is the extinction led from the formula $E = \log I_0/I_1$, I_0 : transmission at blank, I_1 : transmission at the nucleus.

Table 1

illuminating area (μ)	$r^2 E$
0.67	5.00
1.50	4.59
whole area	3.89

As shown in Table 1 the values obtained are different from each other by the varied areas of illumination. In the case of whole area the value is markedly low, only 77 per cent of that obtained by illuminating the area of 0.67μ in diameter, in which the whole light passing through the material can be caught by the photo-tube without any interference from the aberrant ray. It is expected that a more accurate value will be obtained by calculating with the mean value of each E , as the DNA in the nucleus is heterogenous in distribution. The mean value of the extinctions on the points ranging along the nuclear diameter can be obtained from the extinc-

tion curve which is obtained from the curve of transmission drawn by autorecording apparatus by taking the light passing through each point of the round nucleus (Figs. 4 and 5). In this case the calculation will be made by one of the following two formulas

$$C_Q = r'^2 E_1 \quad (2)$$

$$C_Q = r' S' / 2 \quad (3)$$

$$\text{where } E_1 = 1/n \sum_{i=0}^n Ei, \quad S' = \int f(x) dx$$

r' : one half of d in Fig. 4.

Ei : optical density in each point.

$f(x)$: extinction curve.

These two formulas mean entirely the same fact, because $r' S' / 2 = r'^2 \cdot S / 2r = r'^2 E_1$.



Fig. 4. A picture of the track of the nucleus stained with Feulgen reaction moved passing on the light-spot (1.5μ in diameter).

As indicated in Table 2, the great value is obtained in the illumination with the spot-light of 1.5μ in diameter, i. e. $r' S' / 2$ gives the greatest value. This just seems to be due to the great value in S' , as the r' is to give the radius of the nucleus and should be constant. Actual calculations, however, show that the greatest value of S' is given in the case illuminated with 0.67μ and the smallest one is that illuminated the whole area. Therefore, the greatest value of S' by illuminating with the spot-light of 1.5μ is due to the greater value of r' . This means that the r' give not the actual length half of the diameter of the nucleus. By observing the end portion of the nuclear area by illuminating with the spot-light the absorption occurs already where the center of the spot-light is still outside of the nucleus, and the true ab-

sorption is not given even in the case where more than one half of the light-spot is in the nuclear area can be understood by the diagram in Figure 6.

Taking into consideration of this error, the true absorption curve must be such one as indicated in Fig. 7, the hacked area on which the true value of DNA contents can be estimated. From this fact, the formula 3 must be corrected as follows:

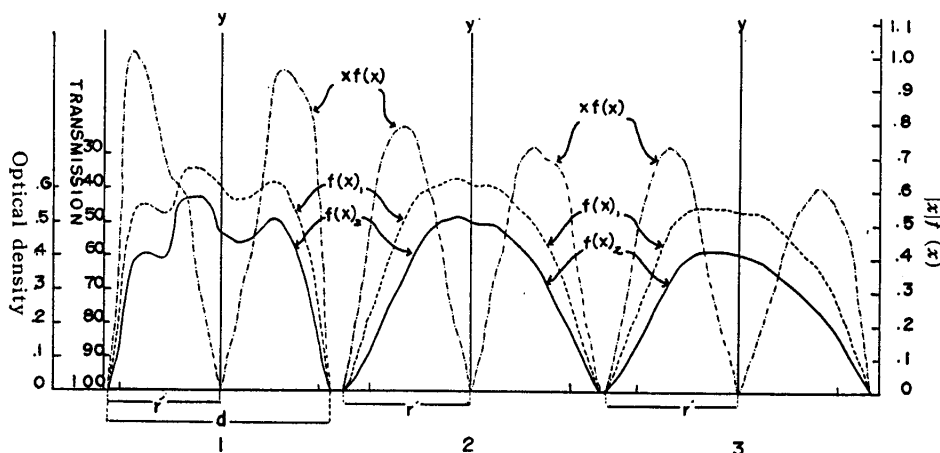


Fig. 5. Curves of transmission $f(x_1)$, extinction $f(x_2)$ and $|x|f(x)$, $f(x_1)$: Transmission curve drawn by autorecording apparatus. $f(x_2)$: Extinction curve obtained from $f(x_1)$. $|x|f(x)$: The curve shows the value of rotating body obtained from the extinction curve by 180° around the y axis. d : Length of the base of transmission curve. r' : One half of d . 1, 2 and 3 show the curves obtained by illuminating with 0.67μ , 1.5μ and the whole area of microscopic field.

Table 2

illuminating area (μ)	$r'S'/2$	S'
0.67	4.03	32.5
1.50	4.65	31.0
whole area	4.32	27.0

$$C_Q = rS/2 \quad (4)$$

where, S : the area surrounded by the curve corrected theoretically.
 r : the radius of the nucleus.

In the calculation by applying the above mentioned formula, the obtained value shows the volume of a cylinder having the base of r'^2 in area and E_1 in height. But the volume of a rotating body of the extinction curve will give a more reliable value. The value of this rotating body is given by the following formula:

$$C_Q = \int_{-r'}^{+r'} |x|f(x)dx \quad (5)$$

In Table 3 the values obtained by the three methods, just mentioned of calculations corrected and non-corrected, are presented summarizing the whole data for the comparison with one another.

As is seen in Table 3 calculated by the corrected curves, the greatest value is always given by the illumination with the smallest spot-light and

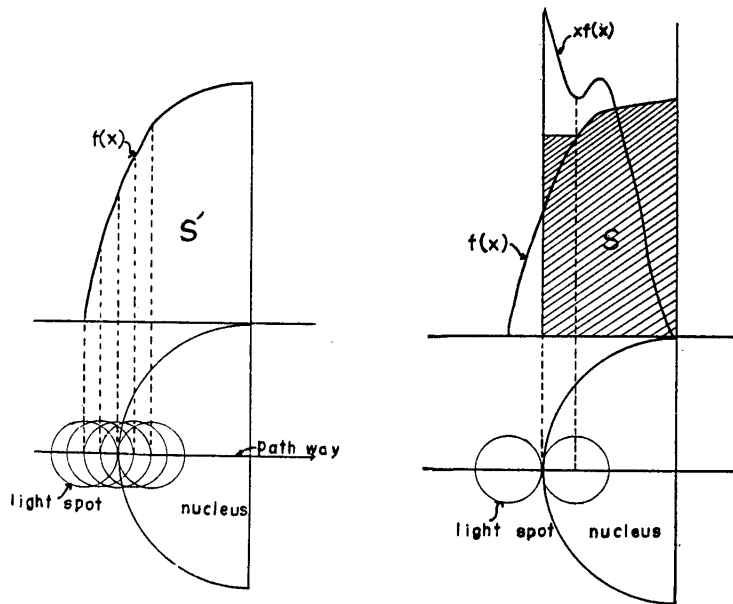


Fig. 6. Diagrammatical drawing showing the relation between the eclipse of spot-light and extinction.

Fig. 7. A schematic diagram showing the method for the theoretical correction of extinction curve: the curve surrounding the oblique line shows the corrected one.

Table 3

illuminating area (μ)	r^2E	$rS/2$		$\int x f(x)dx$	
		non-corrected	corrected	non-corrected	corrected
0.67	5.00	4.03	4.00	3.76	3.76
1.50	4.56	4.65	3.78	3.52	3.52
whole area	3.87	4.32	3.24	3.20	3.20

the smallest value with the illuminating on the whole microscope area; i. e. the same tendency in decrease of the value by calculating with any one of the formulas described.

In the case the calculation is done applying the formula (5), there is no difference between the values obtained from the corrected curves and those from the non-corrected original curves.

COMMENT

As seen in Tables 1, 2 and 3, fairly large values are obtained by calculating with formula r^2E using the value E from the central part of the

nucleus only. These values can be reduced by using E_1 , the mean value of each E found on the nuclear diameter (Table 2. $r'S'/2$), i. e. absolute value of E_1 is always smaller than that of E . This means the heterogenous distribution of the DNA in the nucleus, dense in the central part and less dense in the peripheral part of the nucleus. Therefore, the method to calculate the DNA with the extinction obtained from only one small area of the nucleus should be abandoned, even though this method is very simple in estimation. But as shown repeatedly, the area of illumination on the sample has a great influence to the extinction values, the smallest values in the case illuminating whole area and the largest ones in the case illuminating a small area are, of course, due to the SCHWARZSCHILD-VILLGER effect (S-V effect)^{11,30}, proving that the real value can be obtained only when the area which one wants to measure is illuminated and the whole light passing through this area is introduced into the photo-tube. Thus the results show that the exact values can be obtained when the mean value of each extinction is found along the cell diameter illuminating the small area, 0.67μ in diameter and taking all the light passing through this area into the phototube it is used as a standard for the calculation. Even in this case it is necessary that the mean value of extinction should be led from the extinction curve from the theoretically corrected one instead of the curve obtained by the actual measurement, as explained above.

Now the methods for the calculation the formula (5) must be an ideal one, because this formula shows the volume of a rotating body which is considered to be nearly the same as the total extinction in the whole area of nucleus, whereas the volume of cylinder given by r^2E may rather be far from the total extinction of the nucleus, especially in the case where a great difference is found in the distribution of the substance in the central part and in the peripheral part. And yet, if the formula $\int |x|f(x)dx$ is used for the calculation, there is no difference in the values between those obtained by the curves drawn by actual measurement and those corrected theoretically, showing that the former can be used for the calculation without any correction.

From these facts it can be said that the quantitative estimation of the substance by using the light whose wave length is absorbed specifically to the substance should be done by illuminating only a small area where one wants to measure the substance, introducing the whole light passing through the area into photo-tube, obtaining the extinction curves measuring the extinction in each point along the cell diameter and calculating the value according to the formula $\int |x|f(x)dx$.

Of course, the cells must be kept to contain the whole substance as in the original state throughout the procedure of pretreatments for the measurement. NAORA's method¹¹, which means to measure the E at only one point in the center of the nucleus or the cell, is an ideal one when the nucleus or the cell is globular in shape and applicable for the nucleus found in the tissue sections but there is a difficulty in avoiding those nuclei lying at the section surface where the nuclei are cut off in a part and give incorrect values. Smeared cells however, have all the contents as in the living state when the pretreatment is adequate to avoid the extration of the substance, and will give the exact values.

When one wants to know the absolute value of the DNA or other substances, it is also possible by introducing a constant into the just mentioned formula. The constant can be obtained in two entirely different ways. The first way is to lead it from the contents of some substance (C) and the cell number (N) in a unit volume, as shown in the previous report in the hemoglobin formation in red cell²⁴. i. e. $K_1 = C / N \sum_{i=1}^n E_i$ for the formula (2) or $K_2 = C / N \cdot 1/n \sum_{i=1}^n \int |x| f(x) dx$ for the formula (5). The another way is to obtain the constant from the standard curve drawn with the solution of the purified substance, i. e. $K_3 = C / hE_0$ where C is the concentration of the substance, E_0 is the extinction of the solution measured by MSP, h is the depth of the solution. Then the absolute value of the contents of the substance is calculated as C_0 according to one of the following formulas:

$$C_0 = K_2 \int |x| f(x) dx, \text{ or } C_0 = K_3 \pi \int |x| f(x) dx.$$

A simple method C_0 can also be obtained by the following formulas: $C_0 = K_1 E$, or $C_0 = K_2 \pi r S / 2$. But in this case some error must be taken into consideration as described above.

But of course, the pretreatment must be done carefully so that the substance to be measured may not be lost by extraction or brought to a heterogenous distribution during the course of treatment. If a dye is used for the estimation, the dye from the same company must be used throughout the experiment, with the test that the dye combined with the substance in a quantitative rate or the staining method must be divided so as the dye to combine with the substance in a quantitative rate, controlling the conditions of pretreatment and staining. A little carelessness in the pretreatments often makes it impossible to measure the value of the substance by MSP especially in the case where some dye is used for the staining as suggested by DABIDSON³¹. As for the immersion fluid to be used such a

fluid must be selected as to have its reflective index lying near that of the sample³²

SUMMARY

Some critical experiments have been carried out on the microspectrophotometry using the lymphocytes of a mouse, stained with Feulgen reaction, revealing that most reliable value can be attained by illuminating the material with a small spot-light and integrating the area surrounded by the extinction curve drawn by tracing along the diameter of the smeared and fixed cell.

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